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Force triggers YAP nuclear entry by mechanically regulating transport across nuclear pores

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Highlights:

- ECM-nuclear mechanical coupling drives YAP nuclear translocation in response to substrate rigidity.
- Force application to the nucleus is sufficient for YAP nuclear translocation.
- Force increases YAP nuclear import by decreasing mechanical restriction in nuclear pores.
- Molecular mechanical stability regulates force-dependent transport across nuclear pores.

Summary

YAP is a mechanosensitive transcriptional activator with a critical role in cancer, regeneration, and organ size control. Here we show that force applied to the nucleus directly drives YAP nuclear translocation by decreasing the mechanical restriction of nuclear pores to molecular transport. We demonstrate that the nucleus only connects mechanically to the cytoskeleton above a threshold in substrate rigidity, allowing forces exerted through focal adhesions to reach the nucleus. This leads to nuclear flattening, which increases YAP nuclear import by decreasing the mechanical restriction of nuclear pores to molecular transport. This restriction is further regulated by the mechanical stability of the transported protein. Control of YAP translocation by nuclear force is independent of focal adhesions, the actin cytoskeleton, substrate rigidity, cell-cell adhesion, and the Hippo pathway. Our results unveil a mechanosensing mechanism mediated directly by nuclear pores, demonstrated for YAP but with potential general applicability in transcriptional regulation.

Introduction

Yes Associated Protein (YAP) is a mechanosensitive transcriptional regulator with a major role in cancer and other diseases (Moroishi et al., 2015; Plouffe et al., 2015; Zanconato et al., 2016), development (Porazinski et al., 2015; Varelas, 2014), and organ size control (Zhao et al., 2010). Both biochemical and mechanical cues control YAP's main regulatory mechanism, which is its localization either in the cytoplasm or the nucleus, where it binds to and activates TEAD transcription factors (Zhao et al., 2008). At the biochemical level, YAP is regulated by the Hippo signaling pathway, largely through its phosphorylation (Meng et al., 2016). At the mechanical level, YAP is regulated by mechanical cues such as extracellular matrix (ECM) rigidity, strain, shear stress or adhesive area (Aragona et al., 2013; Benham-Pyle et al., 2015; Calvo et al., 2013; Chaudhuri et al., 2016; Dupont et al., 2011; Elosegui-Artola et al., 2016; Nakajima et al., 2017; Wada et al., 2011). This mechanical regulation requires cytoskeletal integrity (Das et al., 2016), and involves different cytoskeletal and adhesive structures. First, myosin contractility (Dupont et al., 2011; Valon et al., 2017) and actin severing and capping proteins (Aragona et al., 2013), which respectively increase and reduce YAP nuclear localization. Second, the integrin adaptor protein talin, which unfolds under force and leads to YAP nuclear translocation (Elosegui-Artola et al., 2016). Finally, the Linker of the Nucleoskeleton and Cytoskeleton (LINC) complex, the impairment of which decreases nuclear YAP concentration (Driscoll et al., 2015). Further, this mechanical regulation also depends on nuclear transport, since YAP localizes to the nucleus irrespective of mechanical cues when active nuclear export is blocked (Dupont et al., 2011). However, the role of those different molecular elements is unclear, and how mechanical signals regulate YAP remains unknown.

Results

Mechanical coupling between the cytoskeleton and the nucleus is required for YAP nuclear translocation independently of the Hippo pathway.

To start exploring how force regulates YAP, we noted that among the different cytoskeletal structures involved, talin and the LINC complex are particularly relevant for intracellular mechanical connections. Indeed, talin unfolding leads to focal adhesion and stress fiber formation (Elosegui-Artola et al., 2016; Zhang et al., 2008), whereas the LINC complex mechanically couples the nucleus to stress fibers (Lombardi et al., 2011). These observations suggest a mechanism for YAP mechanosensing: talin unfolding and the LINC complex could mechanically couple ECM, focal adhesions, cytoskeleton, and nucleoskeleton, allowing forces exerted via the ECM to reach the nucleus and directly drive YAP translocation. To test this hypothesis, we evaluated YAP response to ECM rigidity. We used talin 1-/- mouse embryonic fibroblasts, which exhibit a wild-type phenotype due to overexpression of talin (Roca-Cusachs et al., 2009; Zhang et al., 2008) (henceforth referred to as control cells), and knocked down talin 2 using a short hairpin RNA (shRNA) (Fig. S1A). We seeded cells on top of polyacrylamide gels of different rigidities coated with fibronectin. In previous work (Elosegui-Artola et al., 2016), we showed that actomyosin forces unfold talin only above a rigidity threshold, triggering YAP nuclear translocation. Confirming these previous results, YAP was mainly in the cytoplasm in control cells seeded on soft substrates, whereas above the rigidity threshold of 5kPa the actin cytoskeleton reinforced with stress fibers, and YAP translocated to the nucleus (Fig. 1A-B). However, when talin 2 was depleted, cells lacked stress fibers and YAP remained in the cytoplasm (Fig. 1A-B) independently of rigidity. We then determined if this threshold was related to a mechanical coupling between the cytoskeleton and the nucleus. To this end, we seeded cells on gels and we stretched gels bi-directionally using a previously described stretch device (Casares et al., 2015; Kosmalska et al., 2015). We then measured strain (defined as the ratio between the stretch-induced

length increment and the original length) for both nuclei and cells. We defined the cell/nucleus mechanical coupling as the ratio between nuclear and cellular strain, which would be 1 if the nucleus stretched as much as cells, and 0 if nuclei were insensitive to cell stretch. The nucleus of talin 2 depleted cells was mechanically uncoupled from the rest of the cell independently of the rigidity of the substrate. In contrast, the nucleus of control cells was uncoupled on soft substrates but coupled above a rigidity threshold (Fig. 1C-D). This threshold coincided with that observed for YAP translocation, suggesting that YAP translocation was mediated by forces transmitted to the nucleus.

To verify this possibility, we blocked the LINC complex by transfecting cells with two dominant negative plasmids (EGFP-Nesprin1-KASH and EGFP-Nesprin2-KASH, Fig. S1A) that block the main interaction of the LINC complex, the link between nesprins and sun proteins (Lombardi et al., 2011; Zhang et al., 2001). Unlike talin depletion, blocking the LINC complex did not affect cell-substrate traction forces or focal adhesions (Fig. S1B-E). However, blocking the LINC complex impaired cell/nuclear mechanical coupling on cells seeded on stiff substrates, reducing it to the levels observed on soft substrates (Fig. 1E). The same effect was observed in the translocation of YAP (Fig. 1F-G), its co-factor TAZ (Fig. S1F), and in one of the main downstream effects of YAP activity, cell proliferation (Fig. S1G). As a control, we checked that the role of the LINC complex was not mediated by its reported association with the microtubule cytoskeleton (Stewart and Burke, 2014). Indeed, depolymerizing microtubules with nocodazole had no effect on YAP localization (Fig. S1H,I). Thus, force transmitted through the LINC complex mediates rigidity-dependent YAP nuclear translocation, and downstream effects. To further support this association, we monitored YAP dynamically during cell spreading on a stiff 29 kPa gel after trypsinization. When control cells transfected with EGFP-YAP (Fig. S1J) started spreading, we observed that YAP remained mainly in the cytoplasm while the nucleus continuously rotated, indicating a loose mechanical link to the actin cytoskeleton and the substrate (quantification of nuclear rotation in fig. 1H, and example in fig. 1I) (Kim

et al., 2014). However, at a given time point the rotation of the nucleus dramatically slowed down, coinciding with the onset of YAP nuclear translocation (Fig. 1H-J and Movie S1).

We then carried out different experiments to assess if this mechanical effect was mediated by biochemical regulation of the Hippo pathway. First, we transfected cells with the YAP mutants FLAG-YAP S94A, which prevents YAP binding to the transcription factor TEAD and thereby reduces nuclear localization (Zhao et al., 2008), and FLAG-YAP S127A, which has impaired phosphorylation leading to decreased cytoplasmic retention (Varelas, 2014) (Fig. S1K). As expected, FLAG-YAP S94A and FLAG-YAP S127A decreased and increased YAP nuclear localization on stiff substrates, respectively (Fig. 1K). However, the rigidity threshold triggering YAP nuclear translocation remained unaltered, and could thus not be explained by biochemical regulation. Second, we evaluated the effect of rigidity and LINC blockage on the expression levels of YAP and the upstream YAP regulators MST and LATS, and YAP phosphorylation (Fig. S1L-M). No effects of either rigidity or LINC blockage were observed. Finally, we evaluated YAP localization after overexpressing LATS and MST, both of which promote YAP phosphorylation and thereby YAP cytoplasmic retention (Piccolo et al., 2014). As expected, overexpression of both proteins reduced YAP nuclear localization, but the effect of rigidity was maintained (Fig. S1N,O). Together, these experiments suggest that forces exerted on ECM adhesions reach the nucleus through the actin cytoskeleton and the LINC complex, triggering YAP nuclear entry independently of the Hippo pathway.

Force application to the nucleus is sufficient to translocate YAP to the nucleus.

Next, we sought to determine whether nuclear force drives YAP translocation directly, or requires transmission through the cytoskeleton. To this end, we plated control cells transfected with EGFP-YAP on soft 5 kPa substrates, at a range where the nucleus and the actin cytoskeleton were uncoupled (Fig. 1C). Then, we used Atomic Force Microscopy (AFM) to directly apply a constant force of 1.5 nN to the cell nucleus, using cantilevers with 20 µm spherical tips (large enough to compress the nucleus globally rather

than locally, see methods). Force application to the nucleus increased the nuclear/cytosolic YAP ratio, which remained constant during force application (Fig. 2A,D and Movie S2), and returned to initial values upon force release (Fig. 2A,D). As a control, nuclear DNA intensity (assessed with a Hoechst dye) remained constant during the experiment, showing that nuclear shape changes induced by force did not affect fluorescence per se (Fig. 2A). No differences in YAP ratio were observed when the spherical probe pressed outside the nucleus, further confirming that YAP translocation was caused specifically by forces applied to the nucleus (Fig. 2C,F and Movie S2). Force-induced YAP nuclear translocation has been proposed to be mediated by actin cytoskeletal integrity (Das et al., 2016), F-actin severing and capping proteins (Aragona et al., 2013) and talin (Elosegui-Artola et al., 2016). To verify the respective roles of these molecular players versus direct nuclear force, we seeded cells on stiff substrates and abrogated YAP nuclear localization, focal adhesions, and cytoskeletal force transmission either by depolymerizing actin with 2 μM cytochalasin D (Fig. S2A-F) or depleting talin (Elosegui-Artola et al., 2016). As expected, both treatments decreased YAP nuclear localization to the levels observed on soft substrates (Fig. S2A-F, and Fig. 1A-B). However, both in the case of cytochalasin D (Fig. 2B,E and Movie S2) and talin depletion (Fig. S2G-H and Movie S3) force application to the nucleus with the AFM was sufficient to rescue YAP nuclear localization.

Force application to the nucleus also induces YAP nuclear localization in confluent cells.

Next, we asked if nuclear forces could also regulate YAP localization in a multicellular context, where high cell density inhibits YAP nuclear translocation (Aragona et al., 2013; Zhao et al., 2007a). To this end, we micropatterned human mammary MCF10A epithelial cells on a 200 μm circular pattern. Within each pattern, we analyzed traction forces exerted on the substrate and YAP localization as a function of the distance to the edge (Fig. 2G-J). As previously described (Bergert et al., 2016), YAP nuclear localization and cell-matrix forces were high at micropattern edges, and decreased at the center of the patterns (Fig. 2I-

J). Consequently, cells with less nuclear YAP exerted lower forces on the substrate (Fig. 2K). Even in this context of strong cell-cell adhesion and low YAP ratios, applying a force with the AFM significantly increased YAP ratios (Fig. 2L-M and Movie S4). Importantly, we note that whereas the response to force was milder than in fibroblasts, this also occurred in isolated MCF10A cells (Fig. S2H-I). Thus, the lower response was due to the different cell type and not the multicellular context. These results demonstrate that force application to the nucleus is sufficient to translocate YAP independently of rigidity, focal adhesions, the actin cytoskeleton, and cell-cell adhesion. Importantly, YAP nuclear translocation is not due to the breakage of the nucleo-cytoplasmic barrier under force, as occurs under very high nuclear deformations (Denais et al., 2016; Raab et al., 2016; Skau et al., 2016). Indeed, such a breakage would not explain the immediate nuclear export observed upon force release. Rather, force disrupts initial YAP distribution, and leads to a new equilibrium that only lasts while force is applied. Therefore, the effects of force application and release may be explained by changes in YAP nuclear import and export kinetics.

Force drives YAP nuclear translocation by increasing active nuclear import.

To test this hypothesis, we interfered with active import and export transport to the nucleus through nuclear pores via two approaches. First, we blocked the function of RAN, a GTPase mediating active import and export to the nucleus (Moore, 1998), through a dominant negative mutant, RAN Q69L (Kazgan et al., 2010). Second, we used leptomycin B, which blocks active export from the nucleus by directly binding to exportin1 (Kudo et al., 1998). On soft substrates, inhibiting all active transport with RAN Q69L had no effect on YAP nuclear localization, but inhibiting only export increased nuclear YAP (Fig. 3A-B). This suggests that import and export are normally balanced on soft substrates. On stiff substrates, Ran Q69L decreased nuclear YAP, but leptomycin B had no effect (Fig. 3A-B). This indicates that import dominates on stiff substrates, either by increasing the import rate or decreasing the export rate. To discriminate between the two options, we carried out Fluorescence Recovery After Photobleaching (FRAP)

experiments to estimate import and export rates on cells transfected with EGFP-YAP (Fig. 3C-D and Movie S5 and S6). To estimate import and export rates, the nucleus or the cytoplasm was bleached, respectively. Once the region of interest was bleached, nuclear fluorescence recovery was quantified. Rates were quantified as the speed of nuclear fluorescence import or export during the initial 30s seconds, divided by the fraction of total YAP available for import or export (respectively in the cytoplasm or nucleus) before bleaching (see methods). As expected, blocking all active transport through RAN Q69L significantly reduced both import and export rates, whereas leptomycin B affected mostly export rates (Fig. S3 and Movie S7 and S8). In control cells, increasing rigidity increased import rates (Fig. 3E, G). In contrast, differences in export kinetics (Fig. 3F) were entirely due to the different nuclear concentrations of YAP on soft/stiff substrates, and rates were not affected (Fig. 3H). This confirms that substrate rigidity promotes YAP nuclear localization by increasing YAP nuclear import. To validate that active nuclear import controls YAP localization, we used the AFM to apply force to the nuclei of cells transfected with Ran Q69L or treated with leptomycin B. As predicted, inhibiting only export with leptomycin B did not prevent force-induced YAP nuclear entry. However, since export was inhibited, releasing force did not restore YAP to the cytoplasm (Fig. 3J-K and Movie S9). In contrast, blocking all transport with RAN Q69L prevented YAP response both to force application and release (Fig. 3I,K and Movie S9). Together, these results confirm that increased active nuclear import is responsible for yap nuclear entry in response to force, either applied directly or by varying rigidity.

Force reduces the mechanical restriction to YAP nuclear translocation exerted by nuclear pores.

We then sought to understand how force affects nuclear import. To this end, we first analyzed how nuclear shape was affected by substrate rigidity, and noticed that nuclei on stiff substrates were more flattened (Fig. 4.B,D). Interestingly, force application with the AFM also leads to nuclear flattening of similar magnitude (see methods). Nuclear flattening may increase nuclear pore permeability: indeed, the

inner lumen of nuclear pores is comprised of a disorganized flexible meshwork of proteins containing phenylalanine-glycine repeats (FG nups) (Frey and Gorlich, 2007; Jamali et al., 2011), which impairs free diffusion (Frey and Gorlich, 2007; Patel et al., 2007; Timney et al., 2016) and exerts mechanical resistance (Bestembayeva et al., 2015). By deforming and flattening the nucleus, force could both partially open pores (reducing mechanical restriction to passage) and increase nuclear membrane curvature. This would increase nuclear pore exposure to the cytosolic versus nuclear side of the membrane, favoring import versus export. To validate this hypothesis and to discriminate flattening from other relevant parameters, we introduced additional perturbations to nuclear shape via hyper and hypo-osmotic shocks. We found that YAP ratio correlated much better with nuclear flattening (Fig. 4A-D) than with any other parameter, including the area, volume, aspect ratio, or height of nuclei, DNA density as assessed with a Hoechst dye, or the ratio between cell and nuclear volume (Fig. S4). Hypo-shocks decreased flattening and YAP ratios at high rigidity, whereas hyper-shocks increased YAP ratios and flattening at low rigidities (where cells and nuclei were mechanically uncoupled).

To confirm the role of nuclear pore mechanical restriction, we carried out different experiments. First, we used transmission electron microscopy (TEM) to measure the size of nuclear pores in cells seeded on soft or stiff substrates. TEM images confirmed that the apparent size of nuclear pores was larger on stiffer substrates (Fig. 4E,F). Second, we perturbed nuclear pore permeability by disrupting FG interactions with trans1-2 cyclohexanediol (CHD) (Ribbeck and Gorlich, 2002) and Pitstop2 (Liashkovich et al., 2015). On soft substrates, increasing nuclear pore permeability with either drug increased YAP ratios, but the effect decreased with increasing rigidity and became negligible at 150kPa (Fig. 4G,H). Finally, we increased the mechanical resistance of YAP to nuclear pore transport by increasing protein size via the addition of one or two EGFP monomers (31 kDa) to the endogenous protein (65 kDa). As predicted, adding one EGFP increased the threshold for YAP nuclear entry from 5 to 15 kPa, and adding two EGFP monomers moved the threshold above the highest rigidity tested (150 kPa) (Fig. 4I,J). Thus, on soft substrates nuclear pores

mechanically restrict YAP import. This restriction is reduced by nuclear force and subsequent flattening on stiff substrates, and increased by molecular weight.

Molecular mechanical stability regulates YAP nuclear translocation.

An interesting property of a system regulated by mechanical restriction to pore transport is that it may depend on protein mechanical stability, as molecules that can easily unfold should oppose less resistance. Certainly, mechanical unfolding of molecules crossing nanometer-size pores occurs during protein degradation, bacterial toxin delivery and protein trafficking between organelles (Olivares et al., 2016; Rodriguez-Larrea and Bayley, 2014; Sato et al., 2005; Thoren et al., 2009). Further, proteins with lower mechanical stability, which unfold more easily, have increased translocation (Berko et al., 2012). Even though the radius of nuclear pores (60 nm, (Beck et al., 2004)) is much larger than those of previously described pores with this feature, the fact that nuclear pores contain FG nups that exert mechanical resistance may lead to a similar effect. To explore this, we first performed single-molecule force spectroscopy AFM experiments at a constant pulling velocity of 400 nm s^{-1} (Neuman and Nagy, 2008) to measure YAP mechanical stability (Fig. 5A). Whereas 41 % of the trajectories exhibited a resistance to unfolding of 60 pN, in the majority of the cases YAP unfolded at undetectable forces (< 10 pN) (Fig. 5B and Fig. S5A-C), thus showing that YAP is *per se* a mechanically labile protein. Then, we tagged YAP with different protein fragments with molecular weights similar or smaller than EGFP, but very well defined mechanical properties. Those are the R16 domain of spectrin, the I27 domain of titin, and the Spy 0128 domain of pilin. R16 and I27 unfold respectively at 30pN (Randles et al., 2007) and 200pN (Carrion-Vazquez et al., 1999; Perez-Jimenez et al., 2006) at similar pulling velocities, and Spy0128 does not unfold even when submitted to the highest force that a single molecule AFM can exert (~800 pN) (Alegre-Cebollada et al., 2010). Remarkably, the rigidity threshold for translocation progressively increased with mechanical stability (Fig. 5C-E). To further isolate the effect of mechanical stability, we generated another

YAP plasmid tagged with I27 V11P, a less mechanically stable one point mutation of I27 (Li et al., 2000) that unfolds at a much lower force of 143 pN. Confirming our hypothesis, YAP I27 V11P translocated to the nucleus between R16 and I27 YAP containing proteins (Fig. 5C-E). Importantly, the differences between the different constructs were largely abolished upon disruption of FG nups with CHD, demonstrating that their differences in nuclear localization were mediated by nuclear pores (Fig. S5B).

Molecular mechanical stability and weight are general regulators of nuclear translocation.

These results place mechanical stability as a novel mechanism to control the molecular specificity of nuclear translocation, and its regulation by force. Such mechanism may be of general applicability beyond YAP, and not even require active transport. To assess this possibility, we studied the localization of the R16, I27 V11P and I27 fragments without YAP. Even in this case, we observed a progressive rigidity threshold for nuclear translocation (Fig. 6A-C). Due to the small size of the fragments (in the absence of YAP), the process was passive rather than active, as the response was not abrogated by RAN Q69L (Fig. S6A). This suggests that the increased exposure of nuclear pores to the cytoplasmic side caused by flattening may be sufficient to promote nuclear import even without active transport. Consistently with this, promoting nuclear import at all rigidities with the addition of a nuclear localization signal (NLS) led to strong nuclear localizations irrespective of rigidity, both for the fragments and for YAP itself (Fig. S6B).

Finally, we checked if this effect of rigidity on the passive nuclear translocation of small proteins was also mediated by nuclear import, as in the case of YAP. To this end, we carried out FRAP experiments on cells seeded on soft or stiff substrates and transfected with different GFP- tagged constructs. To analyze the role of mechanical stability, we used GFP-I27 (with high mechanical stability) and GFP-R16 (with lower mechanical stability). To compare this to the role of molecular weight, we used GFP and a construct with two GFP repeats, 2xGFP. Consistent with our results on YAP, we observed that for all constructs, high rigidity increased import but not export (Fig. 6D-G). Low mechanical stability also increased import, but

did not affect export (Fig. 6D,E). In contrast, increasing molecular weight decreased both import and export (Fig. 6F,G), consistent with impaired overall transport. To further analyze the effect of molecular weight, we measured the progressive nuclear entry of fluorescently labelled dextran molecules of different sizes into the nucleus. Confirming FRAP results, we observed faster nuclear entry on stiffer substrates for all molecular weights (Fig. 6H-K). However and interestingly, the difference between soft and stiff substrates was progressively reduced as molecular weight increased. This shows that whereas nuclear force facilitates the passive transport of large molecules, the effect is diminished as the size of the molecule increases, thereby establishing an optimal protein size for mechanosensitive nuclear transport. In conclusion, both the mechanical stability and the molecular weight of a protein can control nuclear shuttling and localization, independently of biochemical regulation or active transport.

Discussion

Our results unveil the mechanism by which force regulates the translocation of the transcriptional regulator YAP. Even if actin severing proteins (Aragona et al., 2013) and other cytoskeletal structures (Dupont et al., 2011; Elosegui-Artola et al., 2016; Valon et al., 2017) have been suggested to mediate YAP translocation to the nucleus, the actual mechanism that drives YAP into the nucleus was unknown. Here we show that the cytoskeleton and the nucleus only couple mechanically above a threshold in substrate rigidity, allowing cell-ECM forces to reach the nucleus through focal adhesions and the actin cytoskeleton. We demonstrate that force results in nuclear flattening, leading to increased YAP nuclear import due to decreased mechanical restriction to molecular transport in nuclear pores. This mechanical restriction is further regulated by the mechanical stability of the transported molecule, providing a mechanism to control the specificity of force-driven nuclear protein import with potential general applicability (Fig. 7). Indeed, mechanical stability regulated not only the active nuclear import of YAP, but also the passive entry of different short protein fragments. Besides our proposed regulation, we note that additional

mechanisms not directly mediated by nuclear pores could also be at play. For instance, ion channels at the nuclear membrane have been suggested to be mechanosensitive (Ferrera et al., 2014; Finan et al., 2011), and protein concentration gradients could be affected by variations in nuclear/cell volume ratios induced by force. Whereas we can't fully discard a potential role of those mechanisms, we note however that they cannot explain our data. Indeed, hypo-osmotic shocks (which increase membrane tension) had the opposite effect on YAP localization than force or rigidity, and nuclear/cell volume ratios did not correlate with YAP localization (Fig. S4).

Our results place mechanical stability as a novel regulator of nuclear transport in addition to the known contributions of biochemical regulation and protein size (Jamali et al., 2011; Timney et al., 2016). In different types of nanometric pores, recent studies have already highlighted the importance of protein unfolding to allow translocation through narrow openings (Berko et al., 2012; Olivares et al., 2016; Rodriguez-Larrea and Bayley, 2014; Sato et al., 2005; Thoren et al., 2009). In the case of nuclear pores, the forces required for protein unfolding could be mediated by repulsive interactions with FG repeats, which impair molecular diffusion through nuclear pores (Ribbeck and Gorlich, 2002). Supporting this hypothesis, AFM experiments where nuclear pore channels were penetrated with 2 nm tips (and thus molecule-sized) led to repulsive forces of the order of 10^1-10^2 pN, of the same order of the forces required to unfold the different molecules probed in this study (Bestembayeva et al., 2015). Once inside a pore, an unfolded molecule would minimize its interactions with FG repeats by staying in the central part of the pore, thereby facilitating transport.

How translocating molecules interact mechanically with nuclear pore channels, and how this is affected by force-induced conformational changes in nuclear pores, remains as an open question. However, our results suggest the following picture. Repulsive forces from FG-nups unfold a fraction of the proteins entering nuclear pores. This fraction increases as the mechanical stability (unfolding force) of the protein

decreases. However, on soft substrates pores are small enough to impair the transport of proteins even if they are unfolded, and thus protein mechanical stability has no effect. Consequently, there is only slow import, and nuclear/cytosolic ratios are low. As rigidity increases, force is transmitted to the nucleus, the nucleus flattens, and pores gradually open and expose their cytoplasmic side, allowing unfolded proteins to import faster. Since proteins with lower mechanical stability tend to be more unfolded, their translocation is favored. For very stable proteins (such as Spy0128) unfolding never occurs, and thus nuclear localization is low regardless of substrate rigidity.

Our study provides a novel framework to interpret previous findings. First, our results show that talin-mediated mechanosensing allows force transmission from the ECM to the nucleus only above a threshold in substrate rigidity. Thus, any mechanism of direct nuclear mechanosensing can also potentially operate as a rigidity sensor. This includes the control of YAP translocation described here, but also previously described mechanisms of nuclear sensing of forces transmitted through integrins (Tajik et al., 2016) or the LINC complex (Guilluy et al., 2014). Thus, targeting the mechanical connection between the nucleus and the cytoskeleton (i.e., the LINC complex) emerges as a potential approach to interfere with the disruption of homeostasis associated with tissue stiffening in different diseases (Humphrey et al., 2014; Kumar and Weaver, 2009). Second, our results could explain how nucleoskeletal changes affect YAP localization. For instance nuclear softening, which can occur in cancer cells (Cross et al., 2007; Guck et al., 2005), would promote nuclear flattening in response to force, and subsequent YAP nuclear import. Such an effect may be relevant to explain the role of YAP nuclear translocation in malignant transformation (Shimomura et al., 2014). In contrast, overexpression of lamin A leads to a decrease in nuclear YAP (Swift et al., 2013). This could be explained by increased nuclear rigidity (Harada et al., 2014), which would impair nuclear flattening. Finally, developmental scenarios such as gastrulation (Behrndt et al., 2012) or in the inner cell mass (Samarage et al., 2015) involve important cell deformations and shape changes, which likely induce

nuclear deformations. This may provide a mechanism to regulate YAP, which is a fundamental driver in developmental processes.

More generally, our findings reveal a novel mechanosensing mechanism directly converting force into nuclear molecular import. Potentially, this mechanism could regulate the nucleocytoplasmic shuttling of any protein, and control specificity through the mechanical stability of the molecules involved. This may contribute for instance to the localization of other mechanosensitive transcriptional regulators such as MRTF-A (Ho et al., 2013; Zhao et al., 2007b), β-catenin (Fernandez-Sanchez et al., 2015; Mouw et al., 2014) and FHL2 (Nakazawa et al., 2016). Because it directly regulates transcription, this mechanism may be central to influence long-term gene expression in response to mechanical cues, placing force transmission to the nucleus as a fundamental factor.

Contributions: A.E.-A. and P.R.-C. conceived the study, A.E.-A., C.S., X.T., D.N., S.G.-M., and P.R.-C. designed the experiments, A.E.-A., I.A., A.B., M.U., A.K., R.O., J.K., and A-L.R. performed the experiments, A.L., P.R., and S.G.-M. generated reagents, and A.E.-A. and P.R.-C. wrote the paper.

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References

Adam, S.A., Marr, R.S., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol *111*, 807-816.

Alegre-Cebollada, J., Badilla, C.L., and Fernandez, J.M. (2010). Isopeptide bonds block the mechanical extension of pili in pathogenic Streptococcus pyogenes. J Biol Chem *285*, 11235-11242.

Aragona, M., Panciera, T., Manfrin, A., Giulitti, S., Michielin, F., Elvassore, N., Dupont, S., and Piccolo, S. (2013). A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. Cell *154*, 1047-1059.

Bao, Y., Nakagawa, K., Yang, Z., Ikeda, M., Withanage, K., Ishigami-Yuasa, M., Okuno, Y., Hata, S., Nishina, H., and Hata, Y. (2011). A cell-based assay to screen stimulators of the Hippo pathway reveals the inhibitory effect of dobutamine on the YAP-dependent gene transcription. Journal of biochemistry *150*, 199-208.

Basu, S., Totty, N.F., Irwin, M.S., Sudol, M., and Downward, J. (2003). Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell *11*, 11-23.

Bazellieres, E., Conte, V., Elosegui-Artola, A., Serra-Picamal, X., Bintanel-Morcillo, M., Roca-Cusachs, P., Munoz, J.J., Sales-Pardo, M., Guimera, R., and Trepat, X. (2015). Control of cell-cell forces and collective cell dynamics by the intercellular adhesome. Nat Cell Biol *17*, 409-420.

Beck, M., Forster, F., Ecke, M., Plitzko, J.M., Melchior, F., Gerisch, G., Baumeister, W., and Medalia, O. (2004). Nuclear pore complex structure and dynamics revealed by cryoelectron tomography. Science *306*, 1387-1390.

Behrndt, M., Salbreux, G., Campinho, P., Hauschild, R., Oswald, F., Roensch, J., Grill, S.W., and Heisenberg, C.P. (2012). Forces driving epithelial spreading in zebrafish gastrulation. Science *338*, 257-260.

Benham-Pyle, B.W., Pruitt, B.L., and Nelson, W.J. (2015). Cell adhesion. Mechanical strain induces E-cadherin-dependent Yap1 and beta-catenin activation to drive cell cycle entry. Science *348*, 1024-1027.

Berko, D., Tabachnick-Cherny, S., Shental-Bechor, D., Cascio, P., Mioletti, S., Levy, Y., Admon, A., Ziv, T., Tirosh, B., Goldberg, A.L*., et al.* (2012). The direction of protein entry into the proteasome determines the variety of products and depends on the force needed to unfold its two termini. Mol Cell *48*, 601-611.

Bestembayeva, A., Kramer, A., Labokha, A.A., Osmanovic, D., Liashkovich, I., Orlova, E.V., Ford, I.J., Charras, G., Fassati, A., and Hoogenboom, B.W. (2015). Nanoscale stiffness topography reveals structure and mechanics of the transport barrier in intact nuclear pore complexes. Nat Nano *10*, 60-64.

Butler, J.P., Tolic-Norrelykke, I.M., Fabry, B., and Fredberg, J.J. (2002). Traction fields, moments, and strain energy that cells exert on their surroundings. Am J Physiol Cell Physiol *282*, C595-605.

Calvo, F., Ege, N., Grande-Garcia, A., Hooper, S., Jenkins, R.P., Chaudhry, S.I., Harrington, K., Williamson, P., Moeendarbary, E., Charras, G*., et al.* (2013). Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nat Cell Biol *15*, 637-646.

Carrion-Vazquez, M., Oberhauser, A.F., Fowler, S.B., Marszalek, P.E., Broedel, S.E., Clarke, J., and Fernandez, J.M. (1999). Mechanical and chemical unfolding of a single protein: a comparison. PNAS *96*, 3694-3699.

Casares, L., Vincent, R., Zalvidea, D., Campillo, N., Navajas, D., Arroyo, M., and Trepat, X. (2015). Hydraulic fracture during epithelial stretching. Nat Mater *14*, 343-351.

Chaudhuri, O., Gu, L., Klumpers, D., Darnell, M., Bencherif, S.A., Weaver, J.C., Huebsch, N., Lee, H.P., Lippens, E., Duda, G.N.*, et al.* (2016). Hydrogels with tunable stress relaxation regulate stem cell fate and activity. Nat Mater *15*, 326-334.
Creasy, C.L., Ambrose, D.M., and Chernoff, J. (1996). The Ste20-like protein kinase, Mst1, dimerizes and contains an inhibitory domain. J Biol Chem *271*, 21049-21053.
Cross, S.E., Jin, Y.S., Rao, J., and Gimzewski, J.K. (2007). Nanomechanical analysis of cells from cancer patients. Nat Nano *2*, 780-783.
Das, A., Fischer, R.S., Pan, D., and Waterman, C.M. (2016). YAP Nuclear Localization in the Absence of Cell-Cell Contact Is Mediated by a Filamentous Actin-dependent, Myosin II- and Phospho-YAP-independent Pathway during Extracellular Matrix Mechanosensing. The Journal of biological chemistry *291*, 6096-6110.
Denais, C.M., Gilbert, R.M., Isermann, P., McGregor, A.L., te Lindert, M., Weigelin, B., Davidson, P.M., Friedl, P., Wolf, K., and Lammerding, J. (2016). Nuclear envelope rupture and repair during cancer cell migration. Science *352*, 353-358.
Driscoll, T.P., Cosgrove, B.D., Heo, S.J., Shurden, Z.E., and Mauck, R.L. (2015). Cytoskeletal to Nuclear Strain Transfer Regulates YAP Signaling in Mesenchymal Stem Cells. Biophys J *108*, 2783-2793.
Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S.*, et al.* (2011). Role of YAP/TAZ in mechanotransduction. Nature *474*, 179-183.
Elosegui-Artola, A., Bazellieres, E., Allen, M.D., Andreu, I., Oria, R., Sunyer, R., Gomm, J.J., Marshall, J.F., Jones, J.L., Trepat, X.*, et al.* (2014). Rigidity sensing and adaptation through regulation of integrin types. Nat Mater *13*, 631-637.
Elosegui-Artola, A., Oria, R., Chen, Y., Kosmalska, A., Perez-Gonzalez, C., Castro, N., Zhu, C., Trepat, X., and Roca-Cusachs, P. (2016). Mechanical regulation of a molecular clutch defines force transmission and transduction in response to matrix rigidity. Nat Cell Biol *18*, 540-548.
Fernandez-Sanchez, M.E., Barbier, S., Whitehead, J., Bealle, G., Michel, A., Latorre-Ossa, H., Rey, C., Fouassier, L., Claperon, A., Brulle, L.*, et al.* (2015). Mechanical induction of the tumorigenic beta-catenin pathway by tumour growth pressure. Nature *523*, 92-95.
Ferrera, D., Canale, C., Marotta, R., Mazzaro, N., Gritti, M., Mazzanti, M., Capellari, S., Cortelli, P., and Gasparini, L. (2014). Lamin B1 overexpression increases nuclear rigidity in autosomal dominant leukodystrophy fibroblasts. FASEB J *28*, 3906-3918.
Finan, J.D., Leddy, H.A., and Guilak, F. (2011). Osmotic stress alters chromatin condensation and nucleocytoplasmic transport. Biochem Biophys Res Commun *408*, 230-235.
Frey, S., and Gorlich, D. (2007). A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes. Cell *130*, 512-523.
Guck, J., Schinkinger, S., Lincoln, B., Wottawah, F., Ebert, S., Romeyke, M., Lenz, D., Erickson, H.M., Ananthakrishnan, R., Mitchell, D.*, et al.* (2005). Optical deformability as an inherent cell marker for testing malignant transformation and metastatic competence. Biophys J *88*, 3689-3698.
Guilluy, C., Osborne, L.D., Van Landeghem, L., Sharek, L., Superfine, R., Garcia-Mata, R., and Burridge, K. (2014). Isolated nuclei adapt to force and reveal a mechanotransduction pathway in the nucleus. Nat Cell Biol *16*, 376-381.
Harada, T., Swift, J., Irianto, J., Shin, J.W., Spinler, K.R., Athirasala, A., Diegmiller, R., Dingal, P.C., Ivanovska, I.L., and Discher, D.E. (2014). Nuclear lamin stiffness is a barrier to 3D migration, but softness can limit survival. J Cell Biol *204*, 669-682.
Ho, C.Y., Jaalouk, D.E., Vartiainen, M.K., and Lammerding, J. (2013). Lamin A/C and emerin regulate MKL1-SRF activity by modulating actin dynamics. Nature *497*, 507-511.
Humphrey, J.D., Dufresne, E.R., and Schwartz, M.A. (2014). Mechanotransduction and extracellular matrix homeostasis. Nat Rev Mol Cell Biol *15*, 802-812.

Jamali, T., Jamali, Y., Mehrbod, M., and Mofrad, M.R. (2011). Nuclear pore complex: biochemistry and biophysics of nucleocytoplasmic transport in health and disease. Int Rev Cell Mol Biol *287*, 233-286.
Kazgan, N., Williams, T., Forsberg, L.J., and Brenman, J.E. (2010). Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase. Molecular biology of the cell *21*, 3433-3442.
Kim, D.H., Cho, S., and Wirtz, D. (2014). Tight coupling between nucleus and cell migration through the perinuclear actin cap. Journal of cell science *127*, 2528-2541.
Kim, T., Yang, S.J., Hwang, D., Song, J., Kim, M., Kyum Kim, S., Kang, K., Ahn, J., Lee, D., Kim, M.Y.*, et al.* (2015). A basal-like breast cancer-specific role for SRF-IL6 in YAP-induced cancer stemness. Nat Commun *6*, 10186.
Komuro, A., Nagai, M., Navin, N.E., and Sudol, M. (2003). WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. J Biol Chem *278*, 33334-33341.
Kosmalska, A.J., Casares, L., Elosegui-Artola, A., Thottacherry, J.J., Moreno-Vicente, R., Gonzalez-Tarrago, V., del Pozo, M.A., Mayor, S., Arroyo, M., Navajas, D.*, et al.* (2015). Physical principles of membrane remodelling during cell mechanoadaptation. Nat Commun *6*, 7292.
Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E.P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp Cell Res *242*, 540-547.
Kumar, S., and Weaver, V.M. (2009). Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer metastasis reviews *28*, 113-127.
Li, H., Carrion-Vazquez, M., Oberhauser, A.F., Marszalek, P.E., and Fernandez, J.M. (2000). Point mutations alter the mechanical stability of immunoglobulin modules. Nature structural biology *7*, 1117-1120.
Liashkovich, I., Pasrednik, D., Prystopiuk, V., Rosso, G., Oberleithner, H., and Shahin, V. (2015). Clathrin inhibitor Pitstop-2 disrupts the nuclear pore complex permeability barrier. Sci Rep *5*, 9994.
Lombardi, M.L., Jaalouk, D.E., Shanahan, C.M., Burke, B., Roux, K.J., and Lammerding, J. (2011). The interaction between nesprins and sun proteins at the nuclear envelope is critical for force transmission between the nucleus and cytoskeleton. J Biol Chem *286*, 26743-26753.
Meng, Z., Moroishi, T., and Guan, K.L. (2016). Mechanisms of Hippo pathway regulation. Genes Dev *30*, 1-17.
Moore, M.S. (1998). Ran and nuclear transport. J Biol Chem *273*, 22857-22860.
Moroishi, T., Hansen, C.G., and Guan, K.L. (2015). The emerging roles of YAP and TAZ in cancer. Nat Rev Cancer *15*, 73-79.
Mouw, J.K., Yui, Y., Damiano, L., Bainer, R.O., Lakins, J.N., Acerbi, I., Ou, G., Wijekoon, A.C., Levental, K.R., Gilbert, P.M.*, et al.* (2014). Tissue mechanics modulate microRNA-dependent PTEN expression to regulate malignant progression. Nature medicine *20*, 360-367.
Nakajima, H., Yamamoto, K., Agarwala, S., Terai, K., Fukui, H., Fukuhara, S., Ando, K., Miyazaki, T., Yokota, Y., Schmelzer, E.*, et al.* (2017). Flow-Dependent Endothelial YAP Regulation Contributes to Vessel Maintenance. Dev Cell *40*, 523-536 e526.
Nakazawa, N., Sathe, A.R., Shivashankar, G.V., and Sheetz, M.P. (2016). Matrix mechanics controls FHL2 movement to the nucleus to activate p21 expression. PNAS *113*, E6813-E6822.
Neuman, K.C., and Nagy, A. (2008). Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy. Nat Methods *5*, 491-505.
Olivares, A.O., Baker, T.A., and Sauer, R.T. (2016). Mechanistic insights into bacterial AAA+ proteases and protein-remodelling machines. Nat Rev Microbiol *14*, 33-44.
Patel, S.S., Belmont, B.J., Sante, J.M., and Rexach, M.F. (2007). Natively unfolded nucleoporins gate protein diffusion across the nuclear pore complex. Cell *129*, 83-96.

Perez-Jimenez, R., Garcia-Manyes, S., Ainavarapu, S.R., and Fernandez, J.M. (2006). Mechanical unfolding pathways of the enhanced yellow fluorescent protein revealed by single molecule force spectroscopy. J Biol Chem *281*, 40010-40014.
Piccolo, S., Dupont, S., and Cordenonsi, M. (2014). The biology of YAP/TAZ: hippo signaling and beyond. Physiol Rev *94*, 1287-1312.
Plouffe, S.W., Hong, A.W., and Guan, K.L. (2015). Disease implications of the Hippo/YAP pathway. Trends Mol Med *21*, 212-222.
Popa, I., Kosuri, P., Alegre-Cebollada, J., Garcia-Manyes, S., and Fernandez, J.M. (2013). Force dependency of biochemical reactions measured by single-molecule force-clamp spectroscopy. Nat Protoc *8*, 1261-1276.
Porazinski, S., Wang, H., Asaoka, Y., Behrndt, M., Miyamoto, T., Morita, H., Hata, S., Sasaki, T., Krens, S.F., Osada, Y.*, et al.* (2015). YAP is essential for tissue tension to ensure vertebrate 3D body shape. Nature *521*, 217-221.
Raab, M., Gentili, M., de Belly, H., Thiam, H.R., Vargas, P., Jimenez, A.J., Lautenschlaeger, F., Voituriez, R., Lennon-Dumenil, A.M., Manel, N.*, et al.* (2016). ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. Science *352*, 359-362.
Randles, L.G., Rounsevell, R.W., and Clarke, J. (2007). Spectrin domains lose cooperativity in forced unfolding. Biophys J *92*, 571-577.
Ribbeck, K., and Gorlich, D. (2002). The permeability barrier of nuclear pore complexes appears to operate via hydrophobic exclusion. EMBO J *21*, 2664-2671.
Roca-Cusachs, P., Gauthier, N.C., Del Rio, A., and Sheetz, M.P. (2009). Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. PNAS *106*, 16245-16250.
Rodriguez-Larrea, D., and Bayley, H. (2014). Protein co-translocational unfolding depends on the direction of pulling. Nat Commun *5*, 4841.
Samarage, C.R., White, M.D., Alvarez, Y.D., Fierro-Gonzalez, J.C., Henon, Y., Jesudason, E.C., Bissiere, S., Fouras, A., and Plachta, N. (2015). Cortical Tension Allocates the First Inner Cells of the Mammalian Embryo. Dev Cell *34*, 435-447.
Sato, T., Esaki, M., Fernandez, J.M., and Endo, T. (2005). Comparison of the protein-unfolding pathways between mitochondrial protein import and atomic-force microscopy measurements. PNAS *102*, 17999-18004.
Schlierf, M., Li, H., and Fernandez, J.M. (2004). The unfolding kinetics of ubiquitin captured with single-molecule force-clamp techniques. PNAS *101*, 7299-7304.
Serra-Picamal, X., Conte, V., Vincent, R., Anon, E., Tambe, D.T., Bazellieres, E., Butler, J.P., Fredberg, J.J., and Trepat, X. (2012). Mechanical waves during tissue expansion. Nat Phys *8*, 628-634.
Shimomura, T., Miyamura, N., Hata, S., Miura, R., Hirayama, J., and Nishina, H. (2014). The PDZ-binding motif of Yes-associated protein is required for its co-activation of TEAD-mediated CTGF transcription and oncogenic cell transforming activity. Biochem Biophys Res Commun *443*, 917-923.
Skau, C.T., Fischer, R.S., Gurel, P., Thiam, H.R., Tubbs, A., Baird, M.A., Davidson, M.W., Piel, M., Alushin, G.M., Nussenzweig, A.*, et al.* (2016). FMN2 Makes Perinuclear Actin to Protect Nuclei during Confined Migration and Promote Metastasis. Cell *167*, 1571-1585 e1518.
Stewart, C.L., and Burke, B. (2014). The missing LINC: a mammalian KASH-domain protein coupling meiotic chromosomes to the cytoskeleton. Nucleus *5*, 3-10.
Swift, J., Ivanovska, I.L., Buxboim, A., Harada, T., Dingal, P.C., Pinter, J., Pajerowski, J.D., Spinler, K.R., Shin, J.W., Tewari, M.*, et al.* (2013). Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed differentiation. Science *341*, 1240104.

Tajik, A., Zhang, Y., Wei, F., Sun, J., Jia, Q., Zhou, W., Singh, R., Khanna, N., Belmont, A.S., and Wang, N. (2016). Transcription upregulation via force-induced direct stretching of chromatin. Nat Mater *15*, 1287-1296.
Thoren, K.L., Worden, E.J., Yassif, J.M., and Krantz, B.A. (2009). Lethal factor unfolding is the most force-dependent step of anthrax toxin translocation. PNAS *106*, 21555-21560.
Timney, B.L., Raveh, B., Mironska, R., Trivedi, J.M., Kim, S.J., Russel, D., Wente, S.R., Sali, A., and Rout, M.P. (2016). Simple rules for passive diffusion through the nuclear pore complex. J Cell Biol *215*, 57-76.
Valon, L., Marin-Llaurado, A., Wyatt, T., Charras, G., and Trepat, X. (2017). Optogenetic control of cellular forces and mechanotransduction. Nat Commun *8*, 14396.
Varelas, X. (2014). The Hippo pathway effectors TAZ and YAP in development, homeostasis and disease. Development *141*, 1614-1626.
Wada, K., Itoga, K., Okano, T., Yonemura, S., and Sasaki, H. (2011). Hippo pathway regulation by cell morphology and stress fibers. Development *138*, 3907-3914.
Zanconato, F., Cordenonsi, M., and Piccolo, S. (2016). YAP/TAZ at the Roots of Cancer. Cancer Cell *29*, 783-803.
Zhang, Q., Skepper, J.N., Yang, F., Davies, J.D., Hegyi, L., Roberts, R.G., Weissberg, P.L., Ellis, J.A., and Shanahan, C.M. (2001). Nesprins: a novel family of spectrin-repeat-containing proteins that localize to the nuclear membrane in multiple tissues. J Cell Sci *114*, 4485-4498.
Zhang, X., Jiang, G., Cai, Y., Monkley, S.J., Critchley, D.R., and Sheetz, M.P. (2008). Talin depletion reveals independence of initial cell spreading from integrin activation and traction. Nat Cell Biol *10*, 1062-1068.
Zhao, B., Li, L., Lei, Q., and Guan, K.L. (2010). The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev *24*, 862-874.
Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L.*, et al.* (2007a). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev *21*, 2747-2761.
Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Yu, J., Lin, J.D., Wang, C.Y., Chinnaiyan, A.M.*, et al.* (2008). TEAD mediates YAP-dependent gene induction and growth control. Genes Dev *22*, 1962-1971.
Zhao, X.H., Laschinger, C., Arora, P., Szaszi, K., Kapus, A., and McCulloch, C.A. (2007b). Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. J Cell Sci *120*, 1801-1809.

Figure 1. Mechanical coupling between the cytoskeleton and the nucleus is required for YAP nuclear translocation. (A) Quantification of nuclear/cytosolic YAP ratio of control (red) and talin 2 shRNA cells (blue) cells plated on fibronectin-coated polyacrylamide gels of increasing rigidity (n≥20 cells per condition). The dashed line represents the rigidity threshold for YAP translocation to the nucleus. (B) Examples of actin (yellow), Hoechst (cyan) and YAP (grey) stainings for the same conditions. (C) Quantification of the ratio between nuclear and cellular strain for cells stretched on the same conditions as A (n≥11 cells per condition). $p<0.001$ between control cells below and above the threshold for both YAP and stretch experiments. (D) For cells plated on 5 and 29 kPa gels, examples of cell and nuclear shape before and during stretch. Shapes before stretch are shown in gray/blue for cells/nuclei, respectively. Dashed black lines show shapes during stretch. (E) Quantification of nuclear/cellular strain ratios stretched cells on 5 and 29 kPa gels transfected with indicated constructs (n≥15 cells per condition) (F) Nuclear /Cytosolic YAP ratio for the same conditions as E (n≥15 cells per condition). (G) Examples of actin (yellow), Hoechst (cyan) and YAP (grey) stainings for the same conditions. (H) Nuclear rotation speeds for control cells while spreading on fibronectin-coated coverslips (n=19 cells). (I) Representative example of the orientation angle of the nucleus (red) and the nuclear/cytosolic YAP ratio (blue) for a control cell during time while spreading. The dashed line represents the timepoint at which YAP starts entering the nucleus. Reference for the orientation angle is arbitrary. (J) Quantification of nuclear/cytosolic YAP ratio during time for spreading control cells (n=19 cells). (K) Nuclear/cytosolic YAP ratios on gels of increasing rigidity by control cells transfected with indicated constructs (n≥18 cells per condition). $p<0.05$ between cells below and above the threshold for all conditions. ($*p<0.05$,$***p<0.001$). Scale bars are 5 µm for nuclear images in panel D and 20 µm elsewhere. Error bars show standard error of the mean. See also Figure S1 and Movie S1.

Figure 2. Force application to the nucleus is sufficient to translocate YAP to the nucleus. (A) Top: force sequence applied with an AFM cantilever with a 20 μm diameter spherical tip. No force is applied during the first minute, then a 1.5 nN force is applied to the nucleus for 5 minutes, and force stops for the last 4 minutes as the cantilever is retracted. Bottom: Nuclear/cytosolic YAP ratio (red) and Hoechst nuclear average intensity (blue) for control cells seeded on 5kPa gels (n=9 cells) and transfected with EGFP-YAP. (B) Nuclear/cytosolic YAP ratio for the same experimental protocol as in A, but exerting the force on the cytoplasm rather than the nucleus (n=15 cells). (C) Nuclear/cytosolic YAP ratio for the same experimental protocol as in A, in control cells incubated with cytochalasin D seeded on fibronectin coated 29 kPa polyacrylamide gels (n=11 cells). (D-F) Examples of color maps showing YAP fluorescence intensity in the conditions measured respectively in A-C. White arrow in F marks the point of force application. (G-H) Example of YAP staining (G) and color map of traction forces exerted on the substrate (H) by a patterned MCF10A monolayer. (I-J) Average traction forces (I) and nuclear/cytosolic YAP ratios (J) of individual cells as a function of the radial distance from the center of the monolayer (n=348 cells from 13 patterns). (K) Correlation between average traction forces and nuclear/cytosolic YAP ratios of individual cells in the monolayer (n=348 cells from 13 patterns). (L) For the same experimental protocol as in A, nuclear/cytosolic YAP ratio for MCF10A cells inside a monolayer (n=17 cells). (M) Example of color maps showing YAP intensity of MCF10A cells for the experiment measured in L. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Scale bars are 20 μm. Error bars show standard error of the mean. See also Figure S2 and Movie S2, S3 and S4.

Figure 3. Force drives YAP nuclear translocation by increasing active nuclear import. (A) Nuclear/cytosolic YAP ratios of cells plated on gels of 5 and 29kPa. Conditions are: control (red), RAN Q69L transfection (blue) leptomycin B (yellow) (n≥21 cells per condition). (B) Examples of YAP stainings for the conditions in A. (C,D) Examples of FRAP experiments, either the nucleus (C) or the cytoplasm (D) was bleached at t = 0 s in EGFP-YAP transfected cells seeded on 5 or 29 kPa gels. For better visualization, the contrast of images after photobleaching has been adjusted. (E-F) Quantification of nuclear fluorescence after photobleaching the nucleus (E) or cytoplasm (F) (n≥24 and n≥23 cells per condition, respectively). (G-H) Quantification of the import (G) and export (H) rates for the conditions measured in E and F (n≥24 and n≥23 cells per condition, respectively). (I-J) Nuclear/cytosolic YAP ratio for cells incubated with leptomycin B (n=17 cells) (I) or transfected with RAN Q69L (n=16 cells) (J) as nuclear force is applied and released with an AFM tip. (K) Examples of color maps showing YAP intensity for the conditions measured in I,J. Scale bars are 20 µm. (***p <0.001). Error bars show standard error of the mean. See also Figure S3 and Movie S5, S6, S7, S8 and S9.

Figure 4. Force reduces the mechanical restriction to YAP nuclear translocation exerted by nuclear pores. (A-B) Nuclear flattening (A) and nuclear/cytosolic YAP rations (B) of cells on gels of 5, 29 and 150kPa after applying hypo or hyper osmotic shocks (n≥28 and n≥15 cells per condition, respectively). (C) Nuclear flattening versus nuclear/cytosolic YAP ratio for the conditions in A,B. The dashed line shows a linear fit to the data (R^2, squared correlation coefficient). (D) Examples of vertical nuclear sections of cells on 5 and 150 kPa for the conditions measured in B. (E) Nuclear pore diameter in cells on 5/29 kPa gels (n≥41 nuclear pores from ≥19 cells per condition) (F) Corresponding examples of TEM images of nuclear pores. Top insets show magnified images of area marked in red, white arrows show nuclear pores. (G) Nuclear/cytosolic YAP ratios on fibronectin-coated gels of 5, 29 and 150kPa. Conditions are: control (red), CHD incubation (blue) and Pitstop-2 incubation (yellow) (n≥21 cells per condition). (H) Corresponding examples of YAP immunostaining. (I) Quantification of nuclear/cytosolic YAP ratios of cells plated on fibronectin-coated polyacrylamide gels of increasing rigidity. Ratios are shown for endogenous YAP (red), EGFP-YAP (blue), and 2xEGFP-YAP (yellow). $p<0.001$ between all conditions on 29 kPa. (J) Examples of immunostaining on cells plated on 11 and 29 kPa gels for the conditions measured in G. ($*p<0.05$, $**p<0.01$, $***p<0.001$). Scale bars are 20 µm except 10 µm for panel D. Error bars show standard error of the mean. See also Figure S4.

Figure 5. Molecular mechanical stability regulates YAP nuclear translocation. (A) Cartoon depicting single molecule AFM experiments during pulling of individual $(ProteinL)_2$-YAP-$(ProteinL)_2$ polyproteins, composed of a YAP molecule flanked by two ProteinL monomers on each end. (B) Example trace showing polyprotein extension (400 nm s^{-1}) as a function of force. In 59% of the individual trajectories (n=156) the protein elongates by $\Delta L_c \sim 155 \pm 21$ nm (the expected length of the protein) without featuring mechanical stability, followed by the force peaks corresponding to the successive unfolding of the ProteinL monomers (F = 135 pN, ΔL_c = 19 nm), which serve as internal molecular fingerprints. Blue/gray fits show worm-like chain fits to YAP/ProteinL, respectively. These experiments demonstrate that YAP is a mechanically labile protein. (C) Nuclear/cytosolic YAP ratios on gels of increasing rigidity by control cells transfected with indicated constructs (n≥20 cells per condition). Lines show sigmoidal fits to the data. (D) Rigidity threshold for translocation versus unfolding force for each construct in C. Whereas Spy0128 does not translocate or unfold, for representation purposes it is plotted at the maximum tested rigidity (150 kPa) and applied force (800 pN). Significant differences between all conditions were found ($p<0.05$). (E) Examples of FLAG immunostaining on cells plated on 11, 15, 29 and 150 kPa gels for the conditions in C. See also figure S5.

Figure 6. Molecular mechanical stability and weight are general regulators of nuclear translocation. (A) Nuclear/cytosolic ratios of different constructs on gels of increasing rigidity by control cells transfected with different constructs. Constructs are: FLAG-R16 (blue), FLAG-I27 V11P (yellow) and FLAG-I27 (light blue) ($n \geq 20$ cells per condition). Lines show sigmoidal fits to the data. Significant differences between all conditions were found ($p<0.05$). (B) Rigidity threshold for translocation versus unfolding force for each construct in E. (C) Examples of FLAG immunostaining on cells plated on 11, 15 and 29 gels for the conditions in D. Scale bars are 20 μm. (D-G) Nuclear import (D,F) and export (E,G) rates of indicated constructs as obtained from FRAP measurements on cells on 5/29 kPa gels ($n \geq 12$ cells per condition). (H-K) Evolution of nuclear fluorescence after incubating cells on 5/29 kPa gels with fluorescent dextran molecules of different molecular weights ($n \geq 28$ cells per condition). ($*p < 0.05$, $***p < 0.001$). See also figure S6.

Figure 7. Proposed model of mechanosensitive nucleocytoplasmic shuttling. (A) On soft substrates, the nucleus is mechanically uncoupled from the substrate and not submitted to forces. Import and export of YAP through nuclear pores is balanced. (B) On stiff substrates, focal adhesions and stress fibers form, applying forces to the nucleus and flattening it. This stretches and curves nuclear pores, exposing the cytoplasmic side. This leads to increased YAP import. (C) Protein nuclear import depends on molecular weight and mechanical stability: high weight impairs import, and low stability (easily unfolded protein) promotes import. This has potential general applicability beyond YAP.

STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chemicals, Peptides, and Recombinant Proteins		
Critical Commercial Assays		
Deposited Data		
Experimental Models: Cell Lines		

Experimental Models: Organisms/Strains		
Oligonucleotides		
Recombinant DNA		
Software and Algorithms		

Other		

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pere Roca-Cusachs (rocacusachs@ub.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines.

Talin 1 -/- mouse embryonic fibroblasts were described previously (Zhang et al., 2008) and cultured with DMEM 1x (Life technologies 41965) supplemented with 15% FBS, 1% Penicilin, 1% Streptomycin, 1.5 % HEPES. Mammary epithelial MCF10A cells were from ATCC, and grown on DMEM-F12 media supplemented with 5% Horse Serum, 1% Penicillin, 1% Streptomycin, 20 ng/mL EGF, 0.5 mg/mL Hydrocortisone, 100 ng/mL Cholera Toxin, and 10 µg/mL Insulin.

METHOD DETAILS

Antibodies and chemicals

To block active nuclear export, 20 nM Leptomycin B (L2913, Sigma,) was added 3h before starting the experiment. This timeframe maximized nuclear export blockage, while minimizing effects on import. 2µM Cytochalasin D (Sigma) was added 1h before the experiment. Antibodies used for immunostaining were GFP rabbit polyclonal antibody (ab6556, Abcam) and FLAG affinity isolated antibody (F7425, Sigma) to label plasmids containing GFP and FLAG, respectively. mCherry rabbit polyclonal antibody (ab167453, Abcam) was used to recognize mcherry RAN Q69L. To recognize endogenous YAP, YAP mouse monoclonal antibody (clone 63.7 produced in mouse, sc101199, Santa Cruz) was used. Phosphorylated focal adhesion

kinase (Tyr397) was stained with a rabbit polyclonal antibody (44-624G, Thermofisher). Hoechst 33342 (Invitrogen) and Phalloidin–Tetramethylrhodamine B isothiocyanate (Sigma) were used to stain the nucleus and filamentous actin respectively. LATS1 and MST2 were stained with a rabbit monoclonal antibody (3477, Cell Signaling) and a rabbit polyclonal antibody (3682, Cell Signaling), respectively. Antibodies used for western blots were a rabbit polyclonal antibody to detect phosphorylated YAP (Ser127) (4911, Cell Signaling), a mouse monoclonal antibody to recognize Talin (T3287, Sigma) and a mouse monoclonal antibody to detect GAPDH (sc-32233, Santa Cruz). To detect FLAG, GFP, YAP, LATS1 and MST2 in western blots, the same antibodies as for immunostainings were used.

Constructs and transfections.

For stretch experiments, cells were transfected with membrane-targeting plasmid pEYFP-mem (Clontech) described previously (Kosmalska et al., 2015). EGFP-Nesprin1-KASH, EGFP-Nesprin2-KASH and EGFP were described previously (Zhang et al., 2001). EGFP-YAP (Addgene plasmid # 17843), described as pEGFP-C3-hYAP1) (Basu et al., 2003) and FLAG-YAP S127A (Addgene plasmid # 17790, described as p2xFLAGhYAP1-S127A) (Komuro et al., 2003) were a gift from Marius Sudol. FLAG-YAP S94A and FLAG-YAP S94A-NLS were a gift from Dae-Sik Lim (Korea Advanced Institute of Science and Technology, Korea)(Kim et al., 2015). MST2 (Addgene plasmid # 12205, described as pJ3M-Mst2Cherry) was a gift from Jonathan Chernoff (Creasy et al., 1996). LATS1 (Addgene plasmid # 66851, described as pClneoMyc-LATS1) was a gift from Yutaka Hata (Bao et al., 2011). RanQ69L (Addgene plasmid # 30309, described as pmCherry-C1-RanQ69L) was a gift from Jay Brenman (Kazgan et al., 2010). FLAG-R16-YAP, FLAG-I27-YAP, FLAG-I27 V11P-YAP, and FLAG-Spy0128-YAP were generated by amplifying the genes encoding the R16 domain of spectrin, I27 domain of titin (with or without V11P one point mutation), and the Spy0128 domain of Pilin by PCR to add a KpnI restriction site, and subsequently subcloning them into the p2xFLAGhYAP1 vector (Addgene). In the case of 2xEGFP-YAP, the gene was amplified by PCR to add a BglII restriction site and was subsequently inserted into the pEGFP-C3-hYAP1 vector (Addgene). Additional versions of these plasmids were

generated by removing YAP (leaving thus the FLAG tag and the different fragments), with or without adding the Nuclear Localization Signal (NLS) of SV40 large T antigen (CCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCT). 2xGFP, GFP-R16 and GFP-I27 were generated by inserting the corresponding genes into the pEGFP vector. In all experiments involving transfections, the Neon transfection device (ThermoFisher) was used according to manufacturer's instructions. Talin 2 shRNA was used to deplete talin levels as described previously (Elosegui-Artola et al., 2016). All transfections were done the day before the experiment except for talin 2 shRNA experiments that were transfected 5 days before experiment.

Preparation of polyacrylamide gels

Polyacrylamide gels were prepared as described previously (Elosegui-Artola et al., 2014; Elosegui-Artola et al., 2016). Briefly, glass bottom petri dished and slides were activated with a solution of acetic acid, 3-(Trimethoxysilyl)propyl methacrylate (Sigma), and ethanol (1:1:14) for 20 min, washed with ethanol three times and air dried for 10 min. Different concentrations of acrylamide and bis-acrylamide were mixed in a solution to produce gels of different rigidity. The solution contained 2 mg/ml NHS acrylate (Sigma), 0.4% fluorescent far red carboxylated 200 nm beads (Invitrogen), 0.5% ammonium persulfate, and 0.05% tetramethylethylenediamine (TEMED). 10 µl of the solution was then placed on top of the glass and covered with a coverslip. After one hour, the coverslip was removed, and the gels were coated with 10 µg/ml fibronectin (Sigma) overnight at 4 degrees. After washing gels with PBS, cells were trypsinized and seeded on top of gels. Experiments were carried out 4–8 h after cell seeding. The rigidity of polyacrylamide gels was measured with Atomic Force Microscopy as described previously (Elosegui-Artola et al., 2014; Elosegui-Artola et al., 2016).

Immunostaining

For immunostaining, cells were fixed with 4% paraformaldehyde for 15 minutes, washed 3 times with PBS, permeabilized with 0.1% TritonX-100 for 5 minutes, incubated with primary antibodies (1h, room

temperature), and incubated with secondary antibodies (1h, room temperature). Phalloidin was added with the secondary antibodies, whereas Hoechst was added after secondary antibodies for 10 minutes. Fluorescence images were taken with a 60x oil immersion objective (NA=1.40) in an inverted microscope (Nikon Eclipse Ti) or a spinning disk confocal microscope (Andor). The length of pFAK focal adhesions was assessed as described previously (Elosegui-Artola et al., 2016) by measuring the length of bright focal adhesions on the edge of single cells. Nuclear/Cytosolic ratio of YAP, FLAG or GFP was assessed by measuring the intensity of a region of the nucleus and a region with equal size in the cytosol immediately adjacent to the nuclear region. The corresponding Hoechst staining image was used to delimit nuclear versus cytosolic regions.

Western blots

Western blots were implemented following standard procedures. Briefly, cells were lysed using RIPA buffer. Following denaturation, lysates were loaded on 4-20% polyacrylamide gels (Bio-Rad) and transferred onto a nitrocellulose membrane (Whatman, GE Healthcare Life Sciences). After blocking, the membranes were incubated with primary antibody overnight at 4 ºC and with the horseradish-peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature. ECL Western Blotting Substrate (Pierce, ThermoFisher) was used to detect HRP and the bands were visualized with the ImageQuant LAS 400 imaging system (GE Healthcare Life Sciences). The intensity of the bands was analyzed using ImageJ software.

Transmission electron microscopy

For transmission electron microscopy experiments, 5 and 29 kPa polyacrylamide hydrogels were polymerized on top of 12 mm coverslips. Cells were seeded for two hours and fixed with 2,5% glutaraldehyde/1% Paraformaldehyde for 1h at room temperature. Then, cells were post-fixed in the dark with 1% osmium tetroxide 0,8%K4Fe(CN)6 (1 h, room temperature). Following fixation, coverslips were

rinsed with 0.1M Phosphate buffer before being dehydrated in an acetone series (50%, 70%, 90%, 96% and 100%, 10 min each). Coverslips were infiltrated and embedded in Epon (EMS). Blocs were obtained after polymerization at 60ºC for 48 h. 38-40% hydrofluoric acid was used to remove the coverslip. Ultrathin sections of 60 nm in thickness were obtained using a UC7 ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were then stained with 5% uranyl acetate -10' and lead citrate -10'. Sections were observed in a Tecnai Spirit microscope (EM) (FEI, Eindhoven, The Netherlands) equipped with a LaB6 cathode. Images were acquired at 120 kV with a 1376 x 1024 pixel CCD Megaview camera. In images, the nuclear pores seen in cross-section with the nuclear basket visible were selected. Nuclear pore length was measured from one side of the double bilayer, at the point where the nuclear basket (black line) begins, to the other side of the double bilayer where the nuclear basket ends.

Dextran experiments

For dextran nuclear incorporation experiments, cells were permeabilized with 20 µg/ml digitonin (Millipore) for 5 minutes as previously described (Adam et al., 1990; Liashkovich et al., 2015). At this concentration, digitonin permeabilizes the plasma membrane without affecting the nuclear envelope (Adam et al., 1990; Liashkovich et al., 2015). After permeabilization, cells were imaged every 10s with an oil immersion 60x objective (NA=1.40) in an inverted confocal spinning disk microscope (Andor). XX µg/ml of Dextran-FITC (Sigma) of different molecular weights (20, 40, 70 and 150 kDa) were then added. Once background fluorescence stabilized after dextran addition, this was taken as the point of origin and changes in nuclear fluorescence (after background subtraction) were measured as a function of time.

Preparation, procedure and quantification of stretch experiments

Stretch experiments were carried out using a stretch device coupled to an upright Nikon eclipse Ni-U microscope as described before (Casares et al., 2015; Kosmalska et al., 2015). Briefly, stretchable membranes were prepared by mixing PDMS base and crosslinker at a 10:1 ratio, spinning the mixture for

1 minute at 500 rpm, and curing it at 65°C overnight. Once cured, PDMS membraned were peeled off and placed tightly on the stretching device. Then, previously polymerized polyacrylamide gels were pressed on the PDMS membrane and left overnight at 37 °C in a humid chamber. PDMS membranes were previously treated for covalent binding with 3-aminopropyl triethoxysilane, 10% in ethanol for 1 h at 65 °C and with glutaraldehyde (1.5 %) in PBS for 25 min at room temperature. When polyacrylamide gels were bound to the membrane, they were coated with a 10 µg/ml fibronectin solution overnight at 4 °C. After fibronectin coating cells were seeded on the gels, and after 2h membranes were placed on the stretch system. The stretch system has an opening between the central loading post and the external ring. Vacuum is applied through the opening, and deforms and stretches the membrane equibiaxially. For stretch experiments, cells were submitted to 4% linear strain (corresponding to an 8% increase in surface area). Images before and after stretching cells transfected with pEYFP-mem (to delimit the cell) and Hoechst (to delimit the nucleus) were obtained using an upright microscope (Nikon eclipse Ni-U) with a water immersion 60x objective (NA=1.0). For each cell, in order to measure nuclear or cellular strain from images, the length increase of each nucleus and cell was measured.

Atomic Force Experiments and quantification

AFM experiments were carried out in a Nanowizard 4 AFM (JPK) mounted on top of a Nikon Ti Eclipse microscope. Polystyrene beads of 20 µm were attached using a non-fluorescent adhesive (NOA63, Norland Products) to the end of tipless MLCT cantilevers (Veeco). The spring constant of the cantilevers was calibrated by thermal tuning using the simple harmonic oscillator model. Experiments were carried out on cells previously transfected with EGFP-YAP and incubated with Hoechst 33342 (Invitrogen), and seeded on gels in the different conditions described in the results sections. For each cell, the nucleus was identified by using the Hoechst fluorescence signal, and a force of 1.5 nN was applied either to the nuclear region or the cytoplasm (for control experiments). Once the maximum force was reached, the indentation was kept constant for 5 minutes under force control, adjusting the z height by feedback control. After the

5 minutes of indentation, the cantilever was retracted. An image of cell fluorescence (both in the EGFP and Hoechst channels) was captured every minute for 11 minutes (2 before indentation, 5 during indentation and 4 after release) by an Orca ER camera (Hamamatsu) and a 40X (NA = 0.95) objective.

The force applied corresponded to an average final indentation of 1.11 ± 0.3 µm (as measured in n=6 cells, mean ± s.e.m.). As almost all the cell height in this region corresponds to the nucleus (as observed in confocal slices), this indentation corresponds to a change in nuclear flattening (nuclear length/height) from 1.88 to 2.23, in line with the variations caused by rigidity and osmotic shocks in fig. 4C. We note that by applying the Hertz contact model, this indentation corresponds to a diameter of contact between the cell and the spherical probe of 6.7 µm, and a region of deformation which would be somewhat larger but of the same order. This scale of deformation coincides with the measured length scale (~ 10 µm) of nuclei on 5 kPa gels (where most AFM experiments were conducted), thereby providing a mechanical stimulation small enough to selectively deform the nuclear region, but large enough to do so globally.

Osmotic shocks and permeability experiments

Cells were seeded on fibronectin coated gels of 5, 29 and 150kPa for 3h**.** For nuclear permeability experiments, 30 µM Pitstop2 (Abcam) and 258 mM trans-1,2-Cyclohexanediol (Sigma) were added for 1h and 5 minutes, respectively, before fixing the sample with 4% paraformaldehyde. For osmotic shocks, cells were fixed with 4% paraformaldehyde 10 minutes after the shocks. Cell medium has an osmolarity of ~340 mOsm. ~113 mOsm hypo-osmotic shocks (66%) were performed by mixing 1/3 medium with 2/3 de-ionized water. ~695 mOsm Hyper-osmotic shocks (204%) were performed by adding 7.8 µM D-mannitol (Sigma) to the medium. After fixing samples, Immunostaining of YAP and Hoechst was performed and nuclear/cytosolic YAP ratio was measured as described above.

Cell monolayer experiments and quantification

PDMS membranes with 200 µm diameter circular holes were incubated in a 2% Pluronic F-127 (Sigma) solution for 1h. The PDMS membranes were then washed in PBS and air dried for 20 minutes before placing them on previously polymerized 15 kPa polyacrylamide gels. A 10 µg/ml mix of fluorescent fibronectin (Thermo Fisher) and non-fluorescent fibronectin (Sigma) was added to the region with micropatterns and incubated overnight at 4°C. After fibronectin incubation, PDMS membranes were removed and gels were washed. Then, for traction force microscopy measurements, images of the fluorescently labelled fibronectin in circular patterns and underlying embedded fluorescent nanobeads were taken with a 60x oil immersion objective (NA=1.40) on a spinning disk confocal microscope (Andor). Those images provided a reference of the relaxed position of the gel before force application by cells. Then, MCF10A cells were seeded on the micropatterns and left overnight, before obtaining phase contrast images of patterns and of the embedded fluorescent nanobeads. Immediately after image acquisition, cells were fixed with 4% paraformaldehyde. Immunostaining of YAP and Hoechst were performed in order quantify single cells nuclear/cytosolic YAP as described above. Deformation maps were obtained by comparing the fluorescence nanobeads image of patterns with and without cells, using previously described particle image velocimetry software (Serra-Picamal et al., 2012) . From the deformation maps, tractions were inferred assuming that displacements were caused by cells by applying a previously described Fourier Transform algorithm (Bazellieres et al., 2015; Butler et al., 2002).

Fluorescence recovery after photobleaching experiments and quantification

To measure YAP transport in the nucleus, cells were transfected with EGFP-YAP, and plated on 5 or 29kPa polyacrylamide gels 4h before starting the experiment. An area that comprised the nucleus or the cytoplasm was defined in order to bleach the volume contained in that area. The area was bleached with a 488 nm laser (50% power, 2 repeats of 60 µs dwell time) using an inverted confocal spinning disk microscope (Andor). After bleaching, three z-stack images covering 5 µm were acquired with a 60x oil-immersion objective (NA=1.40) to confirm that the entire nuclear volume was affected. For each cell,

images were taken every 300ms during the first 63 seconds, and every 5 seconds (to minimize photobleaching) for 5 more minutes.

For each cell, we calculated the dynamics of nuclear YAP levels as:

$$F = \frac{YAP_{nuc}(t) - YAP_{nuc\ bleach}}{YAP_{intcell}(t) \times c_{ratio}} \times c_{bleach}$$

Where $YAP_{nuc}(t)$ is the average YAP intensity in the nucleus with time, $YAP_{nuc\ bleach}$ is the average YAP intensity of the nucleus in the frame right after the nucleus is bleached, and $YAP_{intcell}(t)$ is the integrated YAP intensity of the cell with time. Dividing by $YAP_{intcell}(t)$ normalizes the results by the overall level of transfection of each cell. Two further correction factors were defined as:

$c_{ratio} = \frac{YAP_{intcell}(prebleach)}{YAP_{intcytosol}(prebleach)}$ (for nuclear bleaching experiments)

$c_{ratio} = \frac{YAP_{intcell}(prebleach)}{YAP_{intnucleus}(prebleach)}$ (for cytosolic bleaching experiments)

$c_{bleach} = \frac{YAP_{intcell}(bleach)}{YAP_{intcell}(t)}$

C_{ratio} is the ratio between the integrated fluorescent intensity of the entire cell, and of the cytosol or nucleus, before bleaching. This parameter is introduced to correct for the fact that the $YAP_{intcell}(t)$ only quantifies either cytosolic or nuclear fluorescence levels (depending on the experiment), because either the nucleus or the cytoplasm has been bleached. C_{bleach} corrects for progressive photobleaching by calculating the ratio between the integrated fluorescence intensity of the cell right after photobleaching, and the same parameter as a function of time. In all cases, background fluorescence was subtracted from fluorescence values before calculations.

The import and export rates were quantified as:

$$Rate = v \times c_{ratio}$$

Where v is the speed of YAP nuclear import or export, obtained as the slope of a first degree polynomial fit to *F* during the first 30 s of measurement. The measurement of v was done during the initial 30 s to best reflect protein import or export only, rather than the balance between the two that occurs after a significant portion of unbleached protein reenters the bleached region. Finally, import/export rates were obtained by multiplying *v* by c_{ratio}, to account for the fact that import/export speeds will be the product of import/export rates times the concentration of protein available for import/export. The import or export rate is measured if the nucleus or the cytosol is bleached, respectively. As an important control, we note that this quantification correctly reproduced the expected changes in import/export rates after treating cells with known inhibitors of nuclear transport (Fig. S3).

Cell spreading experiments

Cells transfected with EGFP-YAP and Hoechst were seeded on 29 kPa polyacrylamide gels. 30 minutes later, the medium was cleaned with PBS and replaced, and gels were placed on an inverted microscope (Nikon Eclipse Ti) with a 40x objective (NA=0.95). Images were taken every 3 minutes while cells were spreading. The level of nuclear YAP was measured as described above. The rotation angle respect to the origin was measured with custom made Matlab software. The software segments the nucleus for every time point, detects the major axis of the nucleus and quantifies the angle of the major axis respect to the origin. The nuclear rotation speed was measured as the absolute value of the angle difference between two consecutive time steps divided by the time step.

Single molecule experiments

To construct the ProteinL_2-YAP-ProteinL_2 polyprotein used in AFM experiments, the YAP gene (Life Technologies) was ligated into the PQE80L vector (Qiagen) using the restriction enzymes BamHI, BglII and KpnI. The recombinant polyprotein was then transformed into *E. coli* BLR (DE)3 cells and grown in LB medium with 100 μg/mL ampicillin at 37°C until an OD_{600} of 0.6 was reached. Cultures were then induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and left overnight at 20°C. Cells were harvested

by centrifugation, resuspended in 50mM phosphate buffer pH 7, 300mM NaCl, 100mg/ml lysozyme, 5 µg/ml Dnase I, 5 µg/ml Rnase A and 10mM MgCl2; and disrupted with a French Press. The protein was then purified by histidine metal affinity chromatography with TALON resin (Takara) and the eluted protein with 250mM imidazole was loaded into a Superdex 200 GL 10/300 column (GE Healthcare) in PBS buffer.

Constant velocity AFM experiments were conducted at room temperature using both a home-made set-up (Schlierf et al., 2004) and a commercial Luigs and Neumann force spectrometer (Popa et al., 2013). In all cases, the sample was prepared by depositing 1–10 µl of protein in PBS solution (at a concentration of 1–10 mg ml^{-1}) on a freshly evaporated gold coverslide. Each cantilever (Si_3N_4 Bruker MLCT-AUHW) was individually calibrated using the equipartition theorem, giving rise to a typical spring constant of ~12–35 pN nm^{-1}. Single proteins were picked up from the surface and pulled at a constant velocity of 400 nm s^{-1}. Experiments were carried out in a sodium phosphate buffer solution, specifically, 50 mM sodium phosphate (Na_2HPO_4 and NaH_2PO_4), 150 mM NaCl, pH=7.2. All data were recorded and analysed using the custom software written in Igor Pro 6.0 (Wavemetrics). For all polyproteins, only recordings showing the signature of at least three events corresponding to the unfolding of the Protein L fingerprint were analysed.

Single cell Traction Force Microscopy measurements

Traction force microscopy measurements were carried out as described previously (Elosegui-Artola et al., 2014; Elosegui-Artola et al., 2016). Briefly, cells were seeded on a gel with a defined rigidity, and placed on an inverted microscope (Nikon Eclipse Ti). Phase contrast images of single cells and fluorescent images of the embedded nanobeads were taken with an 40x (NA=0.6) objective. Then, cells were trypsinized and images of the embedded nanobeads in the relaxed position were taken. Previously described particle image velocimetry (Serra-Picamal et al., 2012) was used to obtain deformation maps comparing bead position in the absence or presence of cells. Then, assuming that deformations were caused by forces

exerted by the cell in the gel, forces maps were inferred using a previously described Fourier transform algorithm (Bazellieres et al., 2015; Butler et al., 2002). The average force for each cell was then measured.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical comparisons were carried out using SigmaStat with two-tailed Student's t-tests when two cases were compared and with analysis of variance (ANOVA) tests when more cases were analyzed. When data did not meet normality criteria, equivalent non-parametric tests were used. Differences were considered to be significant when p values were below 0.05. Details on sample numbers and significance levels are given in figure legends. In all figures, measurements are reported as mean ± standard error of the mean (s.e.m.).